Role of TRPV1 channels, tachykinins and their receptors in anxiety, stress and depression-like behaviour in mice

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ABSTRACT

The tachykinin NK1 receptor was suggested to be involved in psychiatric disorders. Several antagonists have been tested as potential antidepressants, but they failed in the clinical trials. The newest tachykinin, the Tac4 gene-derived hemokinin-1 (HK-1) is present in several brain regions and activates NK1 receptors. We therefore investigated the roles of SP and NKA, HK-1, and NK1 receptors in anxiety, stress and depression-like behaviours in gene-deleted ($Tac1^{-/-}$, $Tac4^{-/-}$, $Tacr1^{-/-}$, respectively) mice compared to C57Bl/6 wildtypes (WT).

Anxiety was evaluated in the light-dark box (LDB) and the elevated plus maze (EPM) where time spent in the dark enclosed area during 20 min and on the closed arms during 5 min was measured, respectively. Spontaneous locomotor activity in the open field test (OFT) was observed during a 5-min session. The sucrose preference test (SPT) is a model for assessing hedonic-anhedonic behaviour, where mice can choose between water and 0.8% sucrose for 2 days. In the tail suspension test (TST) and forced swim test (FST) immobility was assessed during the final 4 min of the 6-min experimental period, referring to the lack of escaping behaviour. Changes of neuronal activity after FST in the $Tac4^{-/-}$ animals was assessed with cFos immunochemistry.

In the LDB $Tac4^{-/-}$ mice spent significantly less time in the lit compartment, while $Tacr1^{-/-}$ animals as well as the NK1 antagonist-treated animals spent significantly more time in the light compartment of the LDB. In the EPM only $Tac4^{-/-}$ showed reduced time on the open arms, no other gene-deletion or treatment with antagonists could influence anxiety level. In the OFT $Tac4^{-/-}$ mice showed significantly less mobility, while the motility of $Tac1^{-/-}$ and $Tacr1^{-/-}$ animals was remarkably greater than that of the WTs, but with pharmacological tools no difference could be detected. $NK1^{-/-}$ consumed markedly more, while $Tac4^{-/-}$ consumed less sucrose solution compared to WTs. In the TST and FST $Tac4^{-/-}$ mice showed remarkably elevated, while $Tacr1^{-/-}$ animals showed decreased immobility in comparison with the WTs,

which was very similar to the treatment with antagonists. Tac4$^{-/-}$ mice showed significantly reduced cFos immunoreactivity after the FST in several stress-related brain regions compared to WTs.

Our study provides the first evidence for an anti-anxiety and stress-coping role of hemokinin-1. These effects are not NK1 receptor-mediated, and the involvement of a putative hemokinin receptor can be proposed. Identification of these targets might open new perspectives for anti-anxiety and anti-depressant therapies.

Abbreviations:

TRPV1 = transient receptor potential vanilloid 1; HK-1 = hemokinin-1; EKA/B/C/D = endokinin A/B/C/D; SP = substance P; Tac1/2/3 = preprotachykinin 1/2/3 gene, NK1/2/3 = tachykinin NK1/2/3 receptor, Tacr1 = tachykinin NK1 receptor encoding gene

1. INTRODUCTION

Mood disorders still belong to the most intensively investigated disorders due to their high and continuously increasing incidence and prevalence as well as the negative impact on the quality of life of impacted families. Furthermore the presently available medical therapy is often without effect or causes severe side effects. The ineffectiveness of these drugs can be interpreted as a consequence of the complex neuronal networks involving different monoamines. The precise pathomechanisms of these disorders remains unknown. Therefore there is an urgent need to unravel the underlying mechanisms and to find new therapeutical targets.

Neuropeptides are widely distributed in the central nervous system (CNS) and the periphery, playing a pivotal regulatory role in several important CNS functions, e.g. thermoregulation, sleep, food intake, memory consolidation (Borbély et al., 2013) and it is beyond doubt that they are important transmitters of stress responses (Kormos et al., 2013). The anti-anxiety properties of substance P (SP), belonging to the tachykinin family, and its preferred tachykinin NK1 receptor have been known for decades. However, as potential anti-depressants, they failed in human studies (Catena-Dell'Osso et al., 2013), and the explanation for this phenomenon remains unclear.

The newest member of the tachykinin family, the Tac4/Ppt-C gene encoding hemokinin-1 (HK-1, in mice and humans) as well as endokinin A-D (in humans) is an interesting and new prospective in the field. HK-1 has a similar structure to SP and shows the highest affinity to NK1 receptors (compared to the presently known NK2 and NK3 receptors). However, HK-1 shows different expression patterns (high concentration in the periphery, especially adrenal glands, and not in the CNS; Duffy, 2005) and also exerts different function from that of SP. Although there are some data describing a role for HK-1 in pain processing (Endo et al., 2006), its involvement in stress responses and mood regulation is presently unknown.

We aimed to investigate the role of Tac1- and Tac4-encoded tachykinins and NK1 receptors in complex behavioural analysis of mice with genetic modifications using pharmacological tools. Furthermore, we determined the effect of the lack of HK-1 on the FST-induced neuronal activation and show for the first time that HK-1 is an important molecule involved in the pathobiology of mood disorders and depression.

(I would state the result in one sentence to end with a strong statement … the above is just an example, but I think something like this would improve your abstract tremendously! ☺)

2. MATERIALS AND METHODS

2.1. Animals

Genetically altered, male TRPV1 and NK1 receptor gene-deleted ($Trpv1^{-/-}$, $Tacr1^{-/-}$), Tac1 and Tac4 gene-deficient ($Tac1^{-/-}$ and $Tac4^{-/-}$) mice were used for the experiments. The original breeding pairs of $Trpv1^{-/-}$ animals were obtained from Jackson Laboratories (USA), $Tac1^{-/-}$ and $Tacr1^{-/-}$ mice from the University of Liverpool, UK (Zimmer et al., 1998; De Felipe et al., 1998). $Tac4^{-/-}$ gene-deficient animals were generated at the Ontario Cancer Institute, Toronto, Canada as previously described (Berger et al., 2010). All types of genetically modified animals were compared to C57Bl/6 mice obtained from Charles-River Ltd. (Hungary) and used as wildtype (WT) controls. Mice weighing 20–25 g were 8–10 weeks old. The animals were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs at 24–25 ˚C, provided with standard mouse chow and water ad libitum and maintained under a 12-h light-dark cycle.

2.2. Investigation of the NK1 receptors with pharmacological tools

C57Bl/6 mice were treated intraperitoneally (i.p.) with the NK1 receptor antagonist CP99994 (25-50 mg/kg, Tocris; dissolved in saline) 30 minutes prior to measurements. As a negative control, one group of animals received the same amount of saline. As a positive control in acute stress tests, one animal group was treated intraperitoneally (i.p.) with citalopram hydrobromide, a selective serotonin reuptake inhibitor (10 mg/kg, Tocris; dissolved in saline) 30 minutes prior to measurements.

2.3. Investigation of acute stress response with forced swim test (FST)

Forced swim test (FST) is a widely used and validated test for the assessment of depression-like behaviour as well as the effect of genetic manipulations related to depression or

antidepressant compounds in rodents (Cryan et al., 2002). During measurements mice react to an inescapable acute stress situation with alternating between struggling and immobility (floating). Animals were placed individually in clear cylinders (height: 25 cm, diameter: 20 cm) containing 19 cm depth of water (24°C). In both tests the total duration of the stress exposure was 6 minutes and the time of immobility referring to the lack of escaping behaviour was measured in the final 4 minutes of the experiment (Porsolt, 1977).

2.4. Investigation of acute stress response with tail suspension test (TST)

Similarly to FST, tail suspension test (TST) is also a commonly used acute stress model of depression-like behaviour. The test is based on the varying ratio of escape-oriented movements and immobile posture and is suitable for the investigation of pharmacological tools with antidepressant activity (Cryan and Mombereau, 2004), as well as the examination of genetical alterations affecting depression-like behaviour (Popova and Tibeikina, 2010). In the TST mice were individually suspended by their tail (distance from the floor was 50 cm) using an adhesive tape at 1 cm from the tip of the tail. TST test was performed in a 6-minute experimental period. The total duration of immobility (complete lack of movement other than respiration) was assessed in the last 4 minutes by a trained observer blinded to the genotypes of the animals (Steru, 1985)

2.5. Investigation of hedonic-anhedonic behaviour with sucrose preference test (SPT)

Sucrose preference test (SPT) is suitable for assessing hedonic-anhedonic behaviour, a core symptom of depression. During this experiment, mice can choose between water and 0.8% sucrose performed on two consecutive days. Mice were placed individually in separate cages where they could choose between two bottles. One bottle contained 0.8% sucrose solution and the second contained tap water. In order to avoid place preference for drinking, the position of

the bottles was changed after 24 h. Food and water were not deprived prior to testing. The level of liquid in each bottle was measured at the same time each day in order to assess sucrose and water consumptions. Sucrose preference was expressed as a percent of total liquid consumed (Jürgenson, 2012).

2.6. Assessment of the anxiety level with elevated plus maze (EPM) test

EPM test is commonly used for the evaluation of anxiety (Lister, 1987). Two opposite open arms (50 cm × 10 cm) and two opposite closed arms (50 cm × 10 cm × 40 cm) make up the equipment, which are located 1 m above the floor. Animals were placed into the central platform and during the 5-min experimental period time spent on the open arms was determined. Data was analysed by EthoVision Basic software (László et al., 2010).

2.7. Investigation of exploratory behaviour (open field test, OFT)

OFT paradigm represents a conflict that arises when the choice is given to explore a new environment and the innate aversion of rodents to brightly lit areas. Mice were placed in a brightly lit wooden box (60 cm x 40 cm) with a floor divided into 16 equal squares (4x4), where the animals can move freely. After placing the animal into the box, the behaviour of the mouse in this novel environment was recorded by a video camera for a 5-minute period. In every case, at the beginning of the measurement, mice were placed into the same corner of the box. Based on the video recording, the following parameters were evaluated: the time spent moving around the box (Holland, 1968).

2.8. Assessment of the anxiety level with light-dark box (LDB) test

Light-dark box test is an anxiety test where light-aversive behaviour contrasts against the tendency to explore a novel environment. LDB tests were performed in a 60 cm × 60 cm × 45

cm (L x W x H) wooden box, consisting of two equally sized compartments. The light compartment was brightly lit, possessing white-painted walls and lacking the top. The dark compartment was not lit, possessing black-painted walls and fully enclosed. The illumination (1000 lx, thermalneutral fiber optic source, Fiber-lite) of the lit compartment uses an intense light source not producing heat. The two compartments are separated by a wall with a 7×7 cm small opening at the floor level. Mice were individually investigated and the time spent in the lit compartment was measured during a 20-minute experimental period (Gaszner et al. 2012).

2.9. c-Fos immunohistochemistry and analysis

Two hours after FST mice were anaesthetized with ketamine (100 mg/kg, i.p) and xylazine (10 mg/kg, i.m.), and perfused with 4% paraformaldehyde. Immunohistochemistry of the brains of C57Bl/6 and Tac4$^{-/-}$ animals was performed as it was previously described (Gaszner et al, 2012). In brief, sections were cut for free-floating diaminobenzidine (Sigma-Aldrich Ltd) immunocytochemistry. Polyclonal antiserum against cFos (Santa Cruz Biotechnology Inc., USA) and goat anti-rabbit IgG were used for the detection of activated neurones.

2.10. Ethics

All experimental procedures were performed according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988) and complied with the recommendations of the International Association for the Study of Pain and the Helsinki Declaration. The studies were approved by the Ethics Committee on Animal Research of University of Pécs according to the Ethical Codex of Animal Experiments and licence was given (licence No.: BA 02/2000-25/2011).

2.11. Statistical analysis

Results are expressed as the mean ± SEM of n = 5–10 mice in each group and analysed with one-way ANOVA followed by Bonferroni's post test. In all cases $p<0.05$ was accepted as statistically significant.

3. RESULTS

3.1. The lack of HK-1 increases immobility time in the FST

C57Bl/6 mice spent approximately 110 sec with immobility, which was similar in Tac1$^{-/-}$ animals. Tacr1$^{-/-}$ mice were remarkably less immobile (50 sec), while Tac4$^{-/-}$ mice were significantly more immobile (160 sec) compared to the WTs (Fig.1A). As a result of saline treatment a slight elevation of immobility could be detected (125 sec), which was decreased by the pre-treatment with citalopram. Furthermore, the treatment with the higher dose of NK1 antagonist, CP99994 reduced the immobility time significantly (57 sec) (Fig.1B).

3.2. HK-1 deficiency increases immobility time in the TST

WT mice spent approximately 170 sec immobile, which was not remarkably altered by the deletion of Tac1 gene. Tacr1$^{-/-}$ animals spent significantly less time immobile (130 sec), while Tac4$^{-/-}$ mice spent more time immobile (190 sec) (Fig.1C). The saline injection increased the immobility time to 185 sec, which was decreased by both (citalopram, CP99994) treatments. The most pronounced reduction (115 sec) was measured after the 50 mg/kg CP99994 NK1 receptor antagonist treatment (Fig.1D).

3.3. Tachykinins are involved in locomotor activity in OFT

WT animals spent 125 sec moving around, which was altered in all gene-deleted groups. Tac1$^{-/-}$ and Tacr1$^{-/-}$ mice moved significantly more (133 sec and 197 sec, respectively) compared to the WTs. The opposite could be observed in the case of HK-1 deficiency: the animals lacking HK-1 spent significantly less time (85 sec) moving (Fig. 2A). Saline treatment reduced the moving time to 100 sec, and CP99994 antagonist treatment increased locomotor time insignificantly (118 sec) (Fig. 2B).

3.4. NK1 receptors and HK-1 influence the time spent in light

C57Bl/6 mice spent 380 sec in the lit compartment of the LDB, which was not altered in Tac1 gene-deficient animals. Time spent in the light was significantly and similarly elevated (545-550 sec) in Tacr1$^{-/-}$ mice, and remarkably reduced in Tac4$^{-/-}$ animals (225 sec; Fig. 2C). The injection of saline itself reduced the time spent in the lit compartment (280) compared to the intact control, and the higher dose of CP99994 significantly increased (580 sec) the time spent in the light compared to their vehicle (Fig.2D).

3.4. Tac4$^{-/-}$ animals show increased anxiety in the EPM test

C57Bl/6 mice spent 60 of the total 300 sec in the open arms of the EPM. Although there were some alterations due to the various gene deletions, the only significant difference could be measured in Tac4$^{-/-}$ animals, which spent significantly less time (40 sec) in the open arms (Fig. 3A). The saline injection influenced anxiety levels (45 sec in open arms) and a significant anti-anxiety effect of citalopram-treatment could be detected (80 sec in open arms). Neither the lower nor the higher dose of CP99994 significantly changed the time spent in the open arms compared to the saline treatment (Fig. 3B).

3.3. Lack of sucrose preference in Tac4$^{-/-}$ animals

The difference between comsuming water or sucrose solution was about 3 ml in WT mice. This amount was slightly but not significantly higher in Tac1$^{-/-}$ animals. In Tacr1$^{-/-}$ mice a pronounced sucrose preference was measured - they consumed 9 ml more sucrose containing water than normal water. On the contrary, Tac4$^{-/-}$ animals did not show sucrose preference at all, the amount of the consumed normal water was higher than the sucrose containing water (Fig.3C).

3.7. The lack of HK-1 decreases neural activity of stress-related brain regions after FST

FST induced significant increase of cFos level in the central, basolateral and medial nuclei of the amygdala (CeA, BLA, MeA), in the oval, dorsolateral, dorsomedial and ventral nuclei of the bed nucleus of stria terminalis (ovBNST, dlBNST, dmBNST, vBNST) also in the dorsal and ventral lateral septum (dLS, vLS), in the parvo- and magnocellular parts of the paraventricular nucleus of the hypothalamus (pPVN, mPVN), as well as in the dorsal and lateral periaqueductal gray matter (dPAG, lPAG) and in the dorsal raphe nucleus (DR) in the WT mice. We could not detect any difference between the control cFos positive nuclei count between the two control groups. However, post-FST Tac4 gene-deleted animals showed significantly less c-Fos positive nuclei in all examined parts of the amygdala (Fig. 4); in both nuclei of the hypothalamus and in the vLS (Fig. 5). Furthermore we can detect a significant decrease in cFos levels in dmBNST and vBNST as well as in the dPAG (Figs. 6, 7). Statistical analysis of the measured cFos values is summarized in Table 1.

4. Discussion

This study reveals a depressed phenotype in HK-1 deficient mice, suggesting that HK-1 exerts antidepressant and stress-coping effects which are the opposite of the classical effect of SP via NK1 receptors. Furthermore we confirm the antidepressant and anxiolytic effects of the NK1 antagonist CP99994, but we can describe only minimal changes in Trpv1 and Tac1 gene-deficient animals. Figures 1 & 2 show clearly that HK-1 and TACR1 ko animals have the exact opposite result and SP ko mice show nothing at all. Fig 3 has big error bars, and only HK-1 ko is significantly reduced, but TACR1 is elevated (albeit not significant) EPM is known to produce huge error bars, a larger number of mice may be needed to achieve statistical significance.

High levels of SP and its preferred receptor NK1 can be found throughout the whole brain, including the stress-related areas (Mantyh, 2002). The fact, that SP and NK1 receptors play an important role in mood regulation and stress coping has been known for decades. There are several data sets addressing the anti-anxiety and antidepressant effect of different NK1 antagonists in distinct animal models: GR 205171 decreased immobility time in FST (Zocchi et al., 2003), FK 888 raised the time spent in the open arms in EPM (Teixeira et al., 1996) in mice and several antagonist – including CP99994 – reduced immobility in TST in gerbils (Varty et al., 2003). Similarly mice lacking NK1 receptors show low anxiety level in FST and TST (Rupniak et al., 2001). In some cases the effects of NK1 antagonists is not the same in males and females or in different strains (Vendruscolo et al., 2003), or the results of $NK1^{-/-}$ animals and the results obtained with NK1 antagonists differs from each other (Rupniak et al., 2001). In our models, the FST, TST, and LDB results of $Tacr1^{-/-}$ animals as well as the high dose (50mg/kg) of NK1 antagonist CP99994 all reveal anti-anxiety/antidepressive effects. Furthermore, mice lacking NK1 receptor showed higher mobility in OFT and increased

sucrose preference, as a sign of improved hedonic behaviour. In $Tac1^{-/-}$ animals we found differences similar to previous data collected by Bilkei-Gorzo et al., (2002). They reported that the deletion of the Tac1 gene leads to decreased anxiety level in FST, TST, and Thatcher-Britton novelty conflict test, and in the OFT mice spent more time in the central field. On the other hand, we found only significant difference in the OFT, where the $Tac1^{-/-}$ animals spent significantly more time with moving.

Little information is available about the possible role of SP in mood disorders: in cerebro-spinal fluid of patients with major depression increased SP level can be measured with radioimmunoassay (RIA) (Bondy et al., 2003) and antidepressant therapy can reduce SP levels (Lieb et al., 2004). But it is important to note, that RIA method is not absolutely specific to SP, since its structure is very similar to HK-1, so it is impossible to distinguish between these two neuropeptides using this technique. Interestingly, the C terminal fragment of SP (SP 6-11: pGlu-Phe-Phe-Gly-Leu-Met-NH_2) was anxiolytic in EPM (Duarte et al., 2004), which is very similar to HK-1 (only one amino acid differs), and this part is responsible for the binding to NK1 receptors (Regoli et al., 1994). There is also data available about the distinct role of C- and N-terminal SP: they have different actions on dopaminergic and serotoninergic pathways in the brain (Pelleymounter et al., 1986), or on the behaviour in EPM after icv. injection (De Araújo et al., 1999). Among the first 7 amino acids of HK-1 only 3 are equivalent with that of SP, which can be a reason for the different effects on behaviour. However, the relevance of SP and NK1 receptors decreased in the last years because NK1 receptor antagonists (e.g.: aprepitant) failed in clinical trials concerning depression therapy (Hafizi et al., 2007). After the successful preclinical results, the reasons for the failure of NK-1 receptor antagonists remain unclear.

Data about the expression pattern and function of HK-1 in the brain are scarce. The cDNA levels of the HK-1-encoding Tac4 gene in the CNS is relatively low compared to Tac1, with

the only exception being the cerebellum. In the periphery the opposite can be observed: Tac4 gene-expression is much higher than Tac1, and very high levels can be measured in the adrenal glands (Duffy et al., 2003), which are an important regulator of stress-responses. Duffy et al. (2003) also reported that central administration of HK-1 induces foot-tapping and scratching behaviour similarly to SP in mice and gerbils, and mediated through NK1 receptors. Additionally, intrathecal administration of hemokinin-1 evokes nociception, which is similar to the SP-induced pain-related behaviours (Watanabe et al., 2010). However, icv. administration of HK-1 in nanomolar concentration exerted analgesic effect in the tail flick test, but in a picomolar concentration it has a significant hyperalgesic effect (Fu et al., 2005). This hyperalgesic effect of HK-1 was verified in a chronic arthritis model (Borbély et al., 2013), utilizing Tac4 gene-deficient animals. Furthermore, SP can decrease the withdrawal latency to noxious heat, which cannot be achieved by addition of HK-1 (Endo et al., 2003). The role of HK-1 was also analysed in pruritus (Funahashi et al., 2014), where it is an important mediator or modulator of the process at spinal cord level, and in IgE-mediated responses, in which it plays a role as an endogenous adjuvant for the inflammatory response (Sumpter et al., 2015), but its role in stress and depression-like behaviour was never investigated. We describe here that Tac4 gene-deficiency lead to stress- and depression-like behaviour in all tests (FST, TST, OFT, LDB, SPT), and that all these alterations are accompanied by significant changes of cFos levels in the important stress-related areas (amygdala, hypothalamus, LS, BNST, PAG). Whether peripheral or central mechanisms (or both) are involved, is not clear.

The first description of HK-1 and its gene (Zhang et al., 2000) suggested that this peptide acted through another receptor and not any of the 3 known NK receptors. Since then, several authors proposed the possibility of a novel receptor for HK-1 on the basis of functional data

which cannot be explained with the presence of the NK1-3 receptors (Endo et al., 2006; Borbély et al., 2013; Funahashi et al., 2014).

In summary, we proved with functional as well as neuronal activation data that HK-1 plays an important role in stress response and mood regulation in mice, which is definitely not NK1 receptor-mediated. Again, I don't agree … I believe this IS NK-1 mediated, but an agonist/antagonist interplay, and thus the opposite effects. We confirmed also, that both genetic deletion and pharmacological inhibition of NK1 receptors with CP99994 have anxiolytic and antidepressant effects, and the same way of influencing TRPV1 receptors leads also to similar effects. Further investigation of the molecular mechanism of these neuropeptides, especially HK-1, may contribute to the better understanding of stress and depression, and may lead to more successful therapy of mood disorders.

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Figure legends

Figure 1. Assessment of anxiety levels in forced swim and tail suspension tests.

Each data point represents the mean ± SEM of immobility time in (**A**) FST in gene-deleted groups of animals, (**B**) FST after pharmacological interventions, (**C**) TST in gene-deleted groups of animals, (**D**) TST after pharmacological interventions (n=8-15 mice per gene-deleted goups and n=5-8 per treated groups; *p<0.05, **p<0.01 ***p<0.001 vs. C57Bl/6 in gene-deleted or vs saline-treated after drug treatment; one-way ANOVA followed by Bonferroni's modified t-test).

Figure 2. Assessment of spontaneous locomotor activity and anxiety in open field and light-dark box tests.

Each data point represents the mean ± SEM of time spent with moving in (**A**) OFT in gene-deleted groups of animals, (**B**) OFT after pharmacological interventions, and time spent in the light in (**C**) LDB in gene-deleted groups of animals, (**D**) LDB after pharmacological interventions (n=8-15 mice per gene-deleted goups and n=5-8 per treated groups; *p<0.05, **p<0.01 ***p<0.001 vs. C57Bl/6 in gene-deleted or vs saline-treated after drug treatment; one-way ANOVA followed by Bonferroni's modified t-test).

Figure 3. **Assessment of anxiety and depression-like behaviour in elevated plus maze and sucrose preference tests**.

Each data point represents the mean ± SEM of time spent on the open arms of (**A**) EPM in gene-deleted groups of animals, (**B**) EPM after pharmacological interventions, and difference of sucrose-containing water and only water consumption in (**C**) SPT in gene-deleted groups of animals (n=8-15 mice per gene-deleted goups and n=5-8 per treated groups; *p<0.05, ***p<0.001 vs. C57Bl/6 in gene-deleted or vs saline-treated after drug treatment; one-way ANOVA followed by Bonferroni's modified t-test).

Figure 4. **Assessment of the role of Tac4 gene-deletion on cFos expression in the amygdala.**

Representative pictures show the control activity of cFos positive neurones in (**A**) C57Bl/6 and (**B**) $Tac4^{-/-}$ animals as well as the increased neuronal activity after FST in (**C**) C57Bl/6 and (**D**) $Tac4^{-/-}$ mice. Each data point represents the mean ± SEM of cFos positive nuclei in the (**E**) central, (**F**) basolateral (**G**) medial amygdala (n=4-6 mice per goup; *p<0.05, ***p<0.001 vs. respective non-stressed controls and $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. stressed C57Bl/6; two-way ANOVA followed by Fischer's post hoc test).

Figure 5. Assessment of the role of Tac4 gene-deletion on cFos expression in the hypothalamus and lateral septum.

Each data point represents the mean ± SEM of cFos positive nuclei in the (**A**) parvocellular and (**B**) magnocellular paraventricular nuclei of the hypothalamus, (**C**) dorsal and (**D**) ventral lateral septum (n=4-6 mice per goup; *p<0.05, ***p<0.001 vs. respective non-stressed controls and $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. stressed C57Bl/6; two-way ANOVA followed by Fischer's post hoc test).

Figure 6. **Assessment of the role of Tac4 gene-deletion on cFos expression in the bed nucleus of stria terminalis.**

Each data point represents the mean ± SEM of cFos positive nuclei in the (**A**) ventral, (**B**) dorsomedial, (**C**) dorsolateral and (**D**) ovale nuclei in the bed nucleus of stria terminalis (n=4-6 mice per goup; **p<0.01, ***p<0.001 vs. respective non-stressed controls and $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. stressed C57Bl/6; two-way ANOVA followed by Fischer's post hoc test).

Figure 7. **Assessment of the role of Tac4 gene-deletion on cFos expression in the periaqueductal gray matter and dorsal Raphe nucleus.**

Each data point represents the mean ± SEM of cFos positive nuclei in the (**A**) dorsal and (**B**) ventral periaqueductal gray matter and the (**C**) dorsal Raphe nucleus (n=4-6 mice per goup;

*p<0.05, ***p<0.001 vs. respective non-stressed controls and $^{\#\#\#}$p<0.001 vs. stressed C57Bl/6; two-way ANOVA followed by Fischer's post hoc test).